

## Persistence of Thymidine Kinase Activity in Mitochondria of a Thymidine Kinase-Deficient Derivative of Mouse L Cells

(5-bromodeoxyuridine/ethidium bromide/mitochondrial DNA/density gradient centrifugation)

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**ABSTRACT** The cell line LM(TK<sup>-</sup>) Cl 1D, a derivative of mouse L fibroblasts deficient in thymidine kinase (EC 2.7.1.21) that shows very little thymidine kinase activity in extracts of whole cells as compared to the parental line, and that does not incorporate thymidine or 5-bromodeoxyuridine into nuclear DNA, has maintained the capacity to incorporate these precursors into mitochondrial DNA at a substantial rate. The amount of [methyl-<sup>3</sup>H]thymidine incorporated into mitochondrial DNA of LM(TK<sup>-</sup>) Cl 1D cells after long-term labeling has been conservatively estimated in different experiments to be between 30 and 60% of that incorporated into mitochondrial DNA or nuclear DNA of strain A9, an L-cell derivative without any thymidine-kinase deficiency; by contrast, the incorporation of thymidine into nuclear DNA of Cl 1D cells is less than 1% of that in A9 cells. These results strongly suggest that the loss of thymidine kinase activity in the extramitochondrial compartment of LM(TK<sup>-</sup>) Cl 1D cells has not been accompanied by the loss of the mitochondria-associated enzyme activity, pointing to a different genetic or epigenetic control of the extramitochondrial and mitochondrial enzymes.

A subline of mouse fibroblasts resistant to BrdU, LM(TK<sup>-</sup>), was isolated by Kit *et al.* (1) by continuous propagation of these cells in medium containing 25  $\mu$ g of BrdU/ml. The cells resistant to BrdU were characterized as deficient in thymidine kinase (EC 2.7.1.21), the enzyme catalyzing the first step in the utilization of thymidine and BrdU (2). Our attempts (3) to isolate closed-circular mitochondrial (mit)DNA from a clonal derivative (Cl 1D) (4) of the LM(TK<sup>-</sup>) line (which was grown routinely in the presence of 30  $\mu$ g of BrdU/ml to prevent reappearance of cells with thymidine kinase activity) met with unexpected difficulties. DNA components were obtained from the mitochondrial fraction of these cells that did not exhibit, in a CsCl-ethidium bromide (EthBr) solution, containing the typical sedimentation pattern of mit-DNA monomeric closed-circular and monomeric open-circular or linear forms. Under the same conditions of isolation, typical closed- and open-circular or linear mit-DNA could, on the contrary, be obtained from the parental L-cell line and from another L-cell derivative, A9 (grown in the absence of BrdU), deficient in hypoxanthine-guanine phosphoribosyl transferase (EC 2.4.2.8; ref. 5). An analysis of the density of the DNA extracted from the mitochondrial fraction of Cl 1D cells in a neutral CsCl gradient revealed a broad distribution of densities in a range considerably higher than the expected density of mouse mit-DNA ( $\rho \simeq 1.69$  g/cm<sup>3</sup>). This observation strongly suggested that BrdU was incorporated into mit-DNA of these cells, in contrast to what had been reported for nuclear DNA of LM(TK<sup>-</sup>) cells grown in the presence of BrdU (1). In fact, after elimination of BrdU from the

growth medium of Cl 1D cells, the extracted mit-DNA exhibited a normal sedimentation behavior in CsCl-EthBr solution, and had a density in neutral CsCl identical to that of mit-DNA from other mouse-cell lines (3).

A plausible interpretation of these results was that thymidine kinase activity is indeed present in mitochondria of Cl 1D cells, despite its reported absence in extracts of whole cells, and that the unusual physical properties of mit-DNA from cells grown in the presence of BrdU were the result of the incorporation of this halogenated precursor and of photochemical nicking. These observations prompted a systematic and quantitative investigation of the capacity of Cl 1D cells to incorporate thymidine and BrdU into mit- and nuclear DNAs. Our results give strong support to the conclusion of persistence of mitochondrial thymidine kinase activity in Cl 1D cells, pointing to a different genetic or epigenetic control of the extramitochondrial and mitochondrial enzymes.

### MATERIALS AND METHODS

**Cells and Media.** The cell lines were grown attached to 250-ml plastic tissue culture bottles or 10-cm Petri dishes in Eagle's medium or in spinners in modified Eagle's medium (6), in both cases with 10% calf serum. The LM(TK<sup>-</sup>) Cl 1D cell line was grown in the dark in the presence or absence of 30  $\mu$ g of BrdU/ml (generation time about 21 hr). The A9 cell line, an L-cell derivative resistant to 8-azaguanine and deficient in hypoxanthine-guanine phosphoribosyl transferase (5), was grown in the presence of 3  $\mu$ g of 8-azaguanine/ml (generation time about 30 hr). Both cell lines were obtained from Dr. Boris Ephrussi, Centre de Génétique Moléculaire du C.N.R.S., Gif-sur-Yvette, France.

**Labeling Conditions.** DNA of Cl 1D cells grown in the presence of BrdU was labeled by exposure of bottled cultures to [<sup>3</sup>H]deoxycytidine (8.75 Ci/mmol; 0.83  $\mu$ Ci/ml) for 48 hr. DNA of Cl 1D cells grown in the absence of BrdU and of A9 cells was labeled by exposure of cultures in Petri dishes or in spinners to [<sup>14</sup>C]adenosine (47.2-58.1 Ci/mol; 0.125-0.187  $\mu$ Ci/ml) for 1-2 cell generations, or to [methyl-<sup>3</sup>H]thymidine (27.8 Ci/mmol added at a concentration of 0.187-0.375  $\mu$ Ci/ml for about 1 generation and again for 1/2-1 generation). In order to study the effect of EthBr on the labeling of mit-DNA of Cl 1D, spinner cultures were exposed to [methyl-<sup>3</sup>H]thymidine (19 Ci/mmol; 1.67  $\mu$ Ci/ml) for 5 hr in the presence or absence of 1  $\mu$ g of EthBr/ml in the dark, and mixed with cells labeled with [<sup>14</sup>C]thymidine (62 Ci/mol; 0.025  $\mu$ Ci/ml) for 36 hr.

**Isolation of mit-DNA.** Mit-DNA from Cl 1D or A9 cells grown in bottles or Petri dishes was isolated by sedimentation-velocity and density-equilibrium centrifugation in CsCl-

Abbreviations: mit-DNA, mitochondrial DNA; EthBr, ethidium bromide.

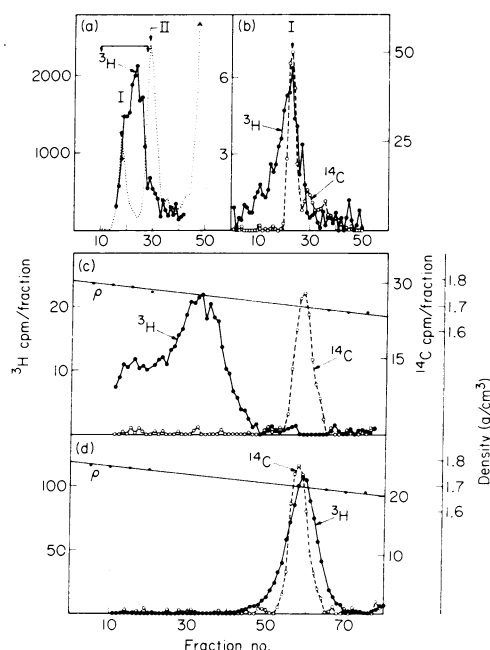


FIG. 1. Incorporation of BrdU into mit-DNA of C1 1D cells. (a) Velocity sedimentation, through a CsCl-EthBr solution, of total mit-DNA from LM(TK<sup>-</sup>) C1 1D cells grown in the presence of BrdU (●—●). The sedimentation profile of total mit-DNA from A9 cells run in a parallel gradient is shown for comparative purposes (.....). (b) Buoyant sedimentation pattern in a CsCl-EthBr density gradient of material from the fractions indicated by arrows in a (●—●); [2-<sup>14</sup>C]thymidine-labeled, closed-circular mit-DNA from HeLa cells (isolated as described in ref. 22) was added as a marker (○—○). (c) and (d) Buoyant densities in CsCl of material from the fractions within brackets in a (c), and of nuclear DNA from LM(TK<sup>-</sup>) C1 1D cells grown in the presence of BrdU (d) (●—●); [2-<sup>14</sup>C]-thymidine-labeled HeLa mit-DNA added as marker (○—○).

EthBr solutions (3). In the isolation of mit-DNA from C1 1D cells labeled in the presence of BrdU the sample was protected as much as possible from light. Mit-DNA from C1 1D and A9 cells grown in spinner cultures was isolated according to Kasamatsu *et al.* (7).

**Isolation of Nuclear DNA.** The nuclear pellets were washed once in 0.25 M sucrose, 10 mM Tris·HCl, pH 6.7 (25°), 1 mM EDTA, and suspended again in 7.5 volumes 0.15 M NaCl, 0.1 M EDTA (pH 7.8), 2% sodium dodecyl sulfate. Nuclear DNA was extracted according to the Marmur procedure (8).

**Analysis of Density of mit-DNA and Nuclear DNA in CsCl Gradients.** CsCl density-gradient centrifugation was performed as described (3), except that, in the case of DNA from C1 1D cells grown in the presence of BrdU, the density of the original CsCl solution was adjusted to 1.73 g/cm<sup>3</sup>.

## RESULTS

### Analysis of mit-DNA and Nuclear DNA from C1 1D Cells Grown in the Presence of BrdU

Fig. 1a shows the sedimentation pattern in a CsCl-EthBr solution of [5-<sup>3</sup>H]deoxycytidine-labeled mit-DNA from LM(TK<sup>-</sup>) C1 1D cells grown in the presence of 30 μg of BrdU/ml. One sees a broad band which, by comparison with the profile of [methyl-<sup>3</sup>H]thymidine-labeled A9 mit-DNA, run

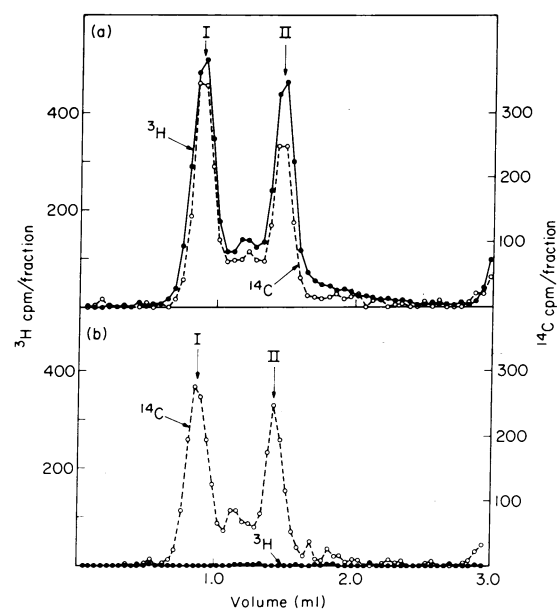


FIG. 2. Incorporation of [methyl-<sup>3</sup>H]thymidine into mit-DNA of LM(TK<sup>-</sup>) C1 1D cells exposed 5 hr to the precursor in the absence (a) or in the presence (b) of 1 μg of EthBr/ml. Equal amounts of cells long-term labeled with [2-<sup>14</sup>C]thymidine were added as an internal standard to each batch of [methyl-<sup>3</sup>H]thymidine-labeled cells. ●—●, <sup>3</sup>H cpm; ○—○, <sup>14</sup>C cpm.

in a parallel tube (Fig. 1a), appears to correspond roughly to the region between monomeric closed-circular (I) and open-circular mit-DNA (II). A portion of the material from this band was run to equilibrium in a CsCl-EthBr density gradient with closed-circular mit-DNA from HeLa cells ( $\rho$  in CsCl-EthBr  $\approx$  1.58) labeled with [2-<sup>14</sup>C]thymidine as a marker (Fig. 1b). It appears that the mit-DNA from C1 1D cells forms in this gradient a fairly broad band extending from  $\rho$  about 1.58 to  $\rho$  about 1.67 g/cm<sup>3</sup>. Another portion of the material from the sedimentation-velocity band was freed of EthBr by Dowex 50 W-X8 chromatography (9), and run to equilibrium in a neutral CsCl density gradient, again with mit-DNA from HeLa cells labeled with [2-<sup>14</sup>C]thymidine as a marker ( $\rho$  about 1.70 g/cm<sup>3</sup>) (Fig. 1c). In this type of gradient, mit-DNA from C1 1D cells grown in the presence of BrdU also appears to be heavier than HeLa mit-DNA and heterogeneous in density, ranging from  $\rho$  about 1.73 to  $\rho$  about 1.78 g/cm<sup>3</sup>; this observation suggests various degrees of substitution of thymidine residues with BrdU. By contrast, the nuclear DNA from the same cells forms in a CsCl density gradient a relatively narrow band with the average density (1.695 g/cm<sup>3</sup>) close to that expected for mouse DNA not substituted with BrdU (1) (Fig. 1d), again suggesting the presence in mitochondria, but not in cytoplasm, of C1 1D of an enzymatic activity allowing the use of BrdU as a DNA precursor.

### Incorporation of [methyl-<sup>3</sup>H]thymidine into mit-DNA of C1 1D Cells with and without EthBr

Fig. 2a and b shows the density profiles in CsCl-EthBr gradients of mit-DNA extracted from two batches of C1 1D cells that had been grown for several generations in the absence of BrdU, then exposed for 5 hr to [methyl-<sup>3</sup>H] thymidine in the absence (a) or in the presence (b) of 1 μg of EthBr/

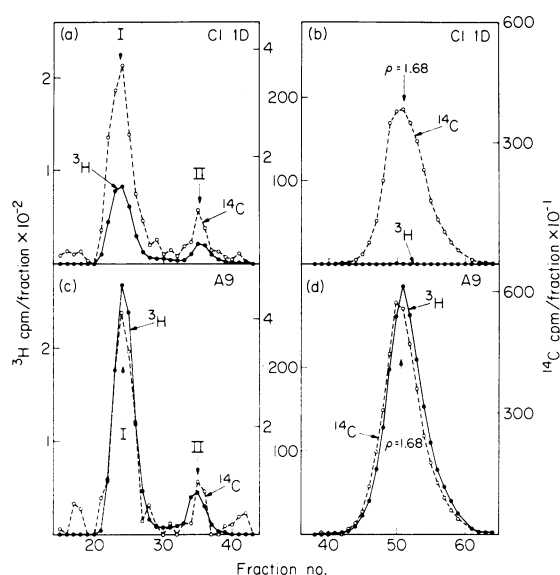


Fig. 3. Long-term incorporation of [methyl- $^3\text{H}$ ]thymidine into mit-DNA and nuclear DNA of LM(TK $^-$ ) Cl 1D and A9 cells. LM(TK $^-$ ) Cl 1D or A9 cells labeled for about 2 generations with [methyl- $^3\text{H}$ ]thymidine were mixed with cells of the corresponding type long-term labeled with [8- $^{14}\text{C}$ ]adenosine. Total mit-DNA was isolated from each mixture and run to equilibrium in a CsCl-EthBr density gradient, as in Fig. 1b; the closed-circular mit-DNA band (I) was sedimented through 1.4 g of CsCl/cm $^3$ -100  $\mu\text{g}$  of EthBr/ml, and then run again in a CsCl-EthBr density gradient (a,c). Nuclear DNA isolated from the same cell mixtures was run in neutral CsCl density gradients (b,d).  $\bullet$ — $\bullet$ ,  $^3\text{H}$  cpm;  $\circ$ — $\circ$ ,  $^{14}\text{C}$  cpm.

ml (added 15 min before the isotope). In order to provide an internal standard for cell breakage and recovery of mit-DNA in the two cell populations, each population was mixed, after harvesting, with an equal amount of Cl 1D cells long-term labeled with [8- $^{14}\text{C}$ ]thymidine. The  $^3\text{H}$  profile of the mit-DNA from untreated cells (a) shows the typical bands, of about equal size, of closed-circular (I) and open-circular or linear mit-DNA (II). Treatment of the cells with EthBr appears to inhibit the labeling of mit-DNA by about 99%, in agreement with results (10) that indicate that 1  $\mu\text{g}/\text{ml}$  of EthBr inhibits by 98% and 95%, respectively, the incorporation of [methyl- $^3\text{H}$ ]thymidine into mit-DNA of HeLa cells and of the mouse-cell line SV 3T3, while inhibiting nuclear DNA (of HeLa and SV 3T3 cells) labeling to a considerably lower degree (about 70% and 50%, respectively).

#### Comparison of the Incorporation of [methyl- $^3\text{H}$ ]thymidine into mit-DNA and Nuclear DNA

In order to compare the incorporation of exogenous [methyl- $^3\text{H}$ ]thymidine in the mitochondrial and extramitochondrial compartments of Cl 1D and A9 cells, a population of Cl 1D cells grown in suspension for several generations in the absence of BrdU and a population of A9 cells were exposed for 2 generations to [methyl- $^3\text{H}$ ]thymidine and mixed, after harvesting, with an appropriate amount of cells of the corresponding type long-term labeled with [8- $^{14}\text{C}$ ]adenosine. Total mit-DNA extracted from each of the two doubly labeled cell populations and run in a CsCl-EthBr density gradient yielded  $^3\text{H}$  radioactivity patterns with well-pronounced bands, of approximately equal size, of closed and

open-circular or linear mit-DNA; on the contrary, the  $^{14}\text{C}$  profiles showed merely hints of mit-DNA bands emerging over a high background, due to small fragments of contaminating RNA and nuclear DNA spread throughout the gradient. The material from the lower band was further purified from these contaminants by sedimentation through a 1.4 g/cm $^3$  CsCl solution in the presence of 100  $\mu\text{g}$  of EthBr/ml, and then run again in a CsCl-EthBr density gradient. The radioactivity patterns of  $^3\text{H}$  and  $^{14}\text{C}$  obtained for the originally closed-circular mit-DNA from the doubly labeled Cl 1D and A9 cell populations are shown, respectively, in Fig. 3a and c. It appears that a minor portion of the closed-circular mit-DNA (I) has been converted to open or linear molecules (II) during the purification procedure. The  $^{14}\text{C}$ -radioactivity data plotted in Fig. 3 have been corrected for differences between the two cell populations in the total amount of  $^{14}\text{C}$  radioactivity originally added to the cells labeled with [methyl- $^3\text{H}$ ]thymidine; the  $^3\text{H}$ -radioactivity values have been normalized to an equal increase of cell mass occurring during the exposure of Cl 1D and A9 cells to [methyl- $^3\text{H}$ ]thymidine. Therefore, the  $^3\text{H}$  to  $^{14}\text{C}$  ratios in the mit-DNA components shown in Fig. 3a and c should reflect the relative incorporation of [methyl- $^3\text{H}$ ]thymidine into mit-DNA of the two cell lines, referred to a unit of long-term [8- $^{14}\text{C}$ ]adenosine label. It appears that Cl 1D cells have incorporated about 40% as much [methyl- $^3\text{H}$ ]thymidine into mit-DNA, per unit of long-term [8- $^{14}\text{C}$ ]adenosine label, as A9 cells. Approximately the same ratio of  $^3\text{H}$  to  $^{14}\text{C}$  was found for the closed-circular and the open-circular or linear mit-DNA components. Mit-DNA from Cl 1D cells labeled with [5- $^3\text{H}$ ]deoxycytidine were reported to have a density identical to that of mit-DNA from A9 or 3T3 mouse-cell lines (3).

In striking contrast to mit-DNA, the incorporation of [methyl- $^3\text{H}$ ]thymidine into nuclear DNA of Cl 1D cells (estimated after normalization of the  $^{14}\text{C}$  and  $^3\text{H}$  radioactivity as explained above) appears to be negligible (Fig. 3b) compared to that observed in A9 cells (Fig. 3d). The slight displacement of the  $^{14}\text{C}$  profile with respect to the  $^3\text{H}$  profile in the DNA from nuclei of A9 cells subjected to buoyant density centrifugation, is presumably due to the partial fractionation on the basis of density, i.e., of GC content, of the nuclear DNA, and to the fact that the [8- $^{14}\text{C}$ ]adenosine labels both deoxyadenylic and deoxyguanylic acid residues, while [methyl- $^3\text{H}$ ]thymidine labels the thymidylic acid residues only. Table 1 summarizes the data of incorporation of [methyl- $^3\text{H}$ ]thymidine into mit-DNA and nuclear DNA of Cl 1D and A9 cells in the experiment described above (Expt. 2), and in two similar experiments. It appears that the incorporation of [methyl- $^3\text{H}$ ]thymidine into mit-DNA of Cl 1D cells, referred to units of long-term [8- $^{14}\text{C}$ ]adenosine label, is from 30 to 60% of that observed in A9 cells, while the labeling of Cl 1D nuclear DNA is less than 1% of that of A9 cells. It is, however, likely that the incorporation of [methyl- $^3\text{H}$ ]thymidine into mit-DNA of Cl 1D cells was underestimated in the present experiments. In fact, the incorporation of [8- $^{14}\text{C}$ ]adenosine into Cl 1D cells, added as an internal standard, per unit of increase of cell mass, was 3–7 times higher than that into A9 cells, at a time when the amount of labeled precursor in the A9 cell culture medium was still far from being exhausted; the labeling of nuclear DNA of Cl 1D cells with [8- $^{14}\text{C}$ ]adenosine was 3–5 times higher than that of A9 cells (Table 1). These observations point to a larger uptake of the labeled precursor

TABLE 1. Incorporation of [methyl-<sup>3</sup>H]thymidine into mit-DNA and nuclear DNA of Cl 1D and A9 cells

	<sup>3</sup> H cpm/ <sup>14</sup> C cpm			<sup>14</sup> C cpm per ml of packed cell volume	<sup>14</sup> C cpm per μg of nuclear DNA increase*
	Mit-DNA I	Mit-DNA II	Nuclear DNA		
<i>Expt. 1</i>					
Cl 1D (a)	1.52	1.37	0.0065	28.2 × 10 <sup>6</sup>	2620
A9 (b)	5.11	4.10	4.78	9.9 × 10 <sup>6</sup>	980
a/b	0.30	0.33	0.0014	2.9	2.7
<i>Expt. 2</i>					
Cl 1D (a)	2.19	2.44	0.014	40.7 × 10 <sup>6</sup>	4590
A9 (b)	4.80	6.81	3.94	11.1 × 10 <sup>6</sup>	1570
a/b	0.46	0.36	0.0035	3.7	2.9
<i>Expt. 3</i>					
Cl 1D (a)	2.19	1.91	0.036	32.9 × 10 <sup>6</sup>	3490
A9 (b)	—	2.94†	4.12	4.8 × 10 <sup>6</sup>	650
a/b	—	0.65	0.0087	6.9	5.4

The <sup>3</sup>H to <sup>14</sup>C ratios for the closed-circular (I) and open-circular or linear (II) mit-DNA components and for nuclear DNA were derived from the experiment illustrated in Fig. 3 and from 2 other similar experiments. (Expt. 1 was performed with cells grown in petri dishes.) The <sup>3</sup>H and <sup>14</sup>C data were normalized as explained in the text.

\* The increase in amount of nuclear DNA during exposure of the cells to [8-<sup>14</sup>C]adenosine was estimated from the final DNA content and from the fractional increase in cell mass.

† In this experiment, the closed-circular mit-DNA from A9 cells isolated by sedimentation velocity through CsCl-EthBr was converted to a great extent to the open-circular or linear forms during the rerun in a CsCl-EthBr density gradient.

and/or to smaller endogenous pools of ATP and dATP in Cl 1D cells compared to A9 cells. In either case, it also seems likely that mit-DNA of Cl 1D cells was 3–5 times more labeled with [8-<sup>14</sup>C]adenosine than mit-DNA of A9 cells. If this is so, the incorporation of [methyl-<sup>3</sup>H]thymidine into mit-DNA of Cl 1D cells, when calculated per unit weight of <sup>14</sup>C-labeled DNA (i.e. corrected for the difference in specific activity of <sup>14</sup>C-labeled DNA), would be similar to that of A9 cells, or even greater.

Although the <sup>3</sup>H to <sup>14</sup>C ratio found in nuclear DNA of Cl 1D cells is very low (Table 1), the total amount of [methyl-<sup>3</sup>H]thymidine label estimated to be present in the various nuclear DNA preparations is from 4 to 25 times more than that found in total mit-DNA in the first sedimentation-velocity or density-equilibrium run; therefore, it is much greater than can be reasonably accounted for by contamination of the nuclear DNA by mit-DNA. This may suggest a certain leakiness of the extramitochondrial thymidine kinase deficiency. However, the errors in <sup>3</sup>H determination in the presence of a large excess of <sup>14</sup>C are such that the significance of the observed small level of apparent <sup>3</sup>H labeling of nuclear DNA is uncertain.

## DISCUSSION

The evidence presented in this paper clearly indicates that the LM (TK<sup>-</sup>) Cl 1D line, an L-cell derivative that has been reported to lack any detectable thymidine kinase activity in extracts of whole cells (1), and that does not incorporate thymidine or BrdU into nuclear DNA (ref. 1 and present work), has preserved the capacity to incorporate these precursors into mit-DNA at a substantial rate. It seems likely that this capacity results from the persistence of thymidine kinase activity in the mitochondria of these cells. The expected low enzyme activity associated with mitochondria, relative to the overall activity present in parental cells, prob-

ably accounts for the failure of earlier investigators to detect it in extracts of whole cells. The normalized incorporation of [methyl-<sup>3</sup>H]thymidine into mit-DNA of Cl 1D cells, was estimated to be from 30 to 60% of that observed in A9 cells, an L-cell derivative without any thymidine kinase deficiency. The experimental variability observed presumably reflects the influence of different conditions of cell growth and labeling used in various experiments. The estimates of the relative capacity of Cl 1D and A9 cells to incorporate exogenous thymidine into mit-DNA are based on the assumption of an equal mit-DNA content of the two cell lines; furthermore, the estimates would obviously be affected by differences between the two cell lines in their uptake of the labeled precursors and/or in the size of their intramitochondrial pools of phosphorylated derivatives of thymidine and adenosine (the latter, because of the use of [8-<sup>14</sup>C]adenosine for the long-term labeling of the cells used as internal standard), and in the level of their thymidylate synthetase activity. As a matter of fact, the observed difference in specific activity of <sup>14</sup>C-labeled nuclear DNA between Cl 1D and A9 cells strongly suggests that the incorporation of [methyl-<sup>3</sup>H]thymidine into mit-DNA of Cl 1D cells was underestimated here with respect to that in A9 cells. In any case, it is justifiable to conclude that the thymidine kinase activity observed in the mitochondria of Cl 1D cells is substantial. In agreement with this conclusion are the results of the analysis of density in a neutral CsCl gradient of BrdU-substituted mit-DNA from Cl 1D cells: these results indicate, in fact, a degree of substitution of thymidine by BrdU ranging from 30 to 90% (11). The heterogeneity in density of BrdU-substituted mit-DNA suggests a variability from cell to cell, or even from mitochondrion to mitochondrion, in mitochondria-associated thymidine kinase activity, and/or in thymidylate synthetase activity.

The sedimentation and density behavior in CsCl-EthBr solutions of BrdU-substituted mit-DNA from Cl 1D cells

suggest that some changes in its physical properties have occurred, presumably as a result of BrdU incorporation. The bulk of the BrdU-substituted mit-DNA, which sediments between the closed and open-circular monomeric forms of unsubstituted mit-DNA in a CsCl (1.4 g/cm<sup>3</sup>)-100  $\mu$ g/ml EthBr solution, and which bands in a CsCl-EthBr density gradient at a somewhat higher density than the closed-circular unsubstituted mit-DNA, may be represented by open dimer molecules. For unsubstituted DNA, this type of molecule has a sedimentation coefficient of 33 S, intermediate between the 37 S and 26 S of the closed and open-circular monomeric forms, respectively (12). On the basis of the increase in density of BrdU-substituted mit-DNA observed in a neutral CsCl gradient (0.04-0.09 g/cm<sup>3</sup>), BrdU-substituted open dimers would be expected to band in a CsCl-EthBr gradient at densities 0.04 to 0.09 g/cm<sup>3</sup> higher than the open-circular monomers of unsubstituted mit-DNA, as the BrdU DNA indeed does. However, electron microscopy of BrdU-substituted mit-DNA from Cl 1D cells will be necessary to ascertain conclusively its physical form.

The basis for the loss of thymidine kinase activity in LM(TK<sup>-</sup>) cells is not known. The stability of this deficiency in the absence of BrdU (1) suggests that a genetic change is involved. In other thymidine kinase-deficient derivatives of L cells (13), the low rate of reversion observed points to a genetic change. The persistence of thymidine kinase activity in mitochondria of LM(TK<sup>-</sup>) Cl 1D cells indicated by the observations reported here may reflect the existence in L cells of a mitochondria-specific enzyme, under the control of nuclear or mitochondrial gene(s), that was not affected, or only partially so, by the change leading to the loss of the extramitochondrial enzymatic activity. However, it is also conceivable that the same enzyme is normally present in the mitochondrial and extramitochondrial compartments, and that the stability or activity of the enzyme in the extramitochondrial compartment has been selectively altered.

There is evidence that isolated mitochondria from chick liver and other sources can incorporate labeled thymidine or BrdU into mit-DNA (14). The results presented here strongly suggest that the capacity of mitochondria to use these deoxyribonucleosides as DNA precursors is not due to the presence of cell-sap enzymes contaminating the mitochondrial preparation, but rather to intrinsic mitochondrial enzymes.

A considerable amount of evidence has associated an increase in thymidine kinase activity in different organisms and cell types with proliferating activity and with an increased rate of DNA synthesis (15); furthermore, in synchronously dividing cells of mammalian cell lines, slime mold, sea urchin embryos, or plants, a periodic increase in thymidine kinase activity has been observed in coincidence with the beginning of DNA replication (15). These observations have suggested a role of this enzyme in the control of DNA synthesis. Since mit-DNA and nuclear DNA exhibit a different temporal pattern of replication (16), the occurrence in the mitochondrial and extramitochondrial compartments of thymidine kinase under different genetic or epigenetic control, as suggested by the present observations, is not surprising if this enzyme has to perform a role in the control of DNA replication. In adrenal glands, whose cells are not actively dividing, most, if not all, of the thymidine kinase activity has been reported to be associated with mitochondria (17); on the contrary, after treatment with adrenocorticotrophic

hormone (ACTH), which stimulates nuclear DNA replication and cell proliferation, there is a marked increase of thymidine kinase activity in the soluble fraction of the cytoplasm (17). These observations are possibly correlated with evidence indicating that mitochondria have a relatively high level of DNA synthesis in resting cells (18, 19).

It will be interesting to investigate the presence of mitochondrial thymidine kinase activity in other "thymidine kinase-less" cell lines, like mouse line 3T3 4E (20) and Chinese hamster line B<sub>14</sub> (21).

Apart from the interest it offers in connection with the above-discussed role of thymidine kinase in the control of DNA synthesis, the persistence in LM(TK<sup>-</sup>) Cl 1D cells of thymidine kinase activity associated with mitochondria, in the absence of extramitochondrial enzyme activity, provides a powerful tool for the selective labeling of mit-DNA with radioactive thymidine or BrdU; furthermore, by the combined use of aminopterin (to block the endogenous synthesis of thymidylic acid) and thymidine, it should be possible to study in this cell line mit-DNA replication under conditions of a selective block of nuclear DNA synthesis.

After this manuscript was submitted for publication, a recent paper (23) by Clayton and Teplitz came to our attention in which they arrive at conclusions qualitatively, but not quantitatively, in agreement with ours.

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